Approvals		
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Removal of Invertebrates from Estuarine and Marine Benthic Samples

1.0. Scope and Application.

1.1 This document describes the procedure used to remove 90% or more of the benthic invertebrates from a benthic sample and sort the animals according to major taxonomic groups. The samples processed have been fixed with 10% formaldehyde and stained with Rose Bengal in the field when collected. This SOP does not include field procedures. When the invertebrates removed with this method are subsequently identified (see SOP R3-QA501) a description of the benthic invertebrate community present at the site sampled is generated and can be used in a bioassessment of water quality. The data is an integral part of biological assessments of estuarine and coastal waters in Region III. Although not yet used in NPDES permits and monitoring, the data is widely used in resource assessment and management by both state and federal agencies.

2.0 Summary of Method

2.1 The fixative (10% formaldehyde) is removed from a sample by decanting, followed by thoroughly washing with tap water that portion of the sample retained on a 500 micrometer sieve. The material retained is spread thinly over the bottom of a large pan and carefully sorted through in order to remove 90% or more of the invertebrates present. The animals removed (or "picked") are transferred to vials of 70% ethanol according to major taxonomic categories (e.g., one vial for crustaceans, bivalves, gastropods, etc.). The sample residue remaining is returned to the field container with fixative. This SOP is adapted from Strobel, et al., 1995.

3.0 Definitions.

3.1 Benthic Invertebrates. The animals to be processed through identification and enumeration will ultimately be determined by a project plan. Typically the benthic invertebrates include all those multicellular animals not belonging to the chordate

subphylum Vertebrata, dwelling on or in the bottom, and retained on a 500 micrometer mesh sieve (or 1.0 millimeter mesh depending on project plan). Excluded are minute taxa that have been traditionally by convention identified as belonging to the meiofauna. These would include nematodes, ostracods, kinorhynchs, tardigrads, harpacticoid copepods, and others.

3.2 Sample. Although in benthic work the term "replicate" is typically used instead of "sample" (a sample being comprised of several replicates), the term "sample" will be used throughout since it is more familiar to those working in a laboratory environment.

4.0 Interferences

Although not an "interference" in the sense of analytical chemistry, problems that can affect the quality of the data produced include the lack of or inconsistent use of Rose Bengal stain, uneven staining (some animals take the stain better than others), incomplete fixation of the animal material, or rough handling in the field of samples on sieves, resulting in fragmented or otherwise damaged specimens that cannot be identified.

5.0 Safety

5.1 All applicable safety and compliance guidelines set forth by the EPA and by federal, state and local regulations must be followed during the performance of this SOP. In addition, all safety procedures described in this SOP and outlined in the Chemical Hygiene Plan (CHP) must be adhered to. All work must be stopped in the event of a known or potential compromise to the health and safety of any associate and/or representative, and the Facility Safety Officer and supervisor immediately notified.

5.2 Material Safety Data Sheets (MSDS) must be maintained in the laboratory for all reagents utilized in the laboratory. This information must be made available to all personnel prior to the performance of this SOP and upon staff request. The MSDSs (hard copies) are currently located in the lab as well as electronically on CD-ROM (software) available in the safety office.

5.3 Analysts will take every precaution to avoid contact with the formaldehyde solution used as a fixative, including breathing the formaldehyde vapors. Formaldehyde is a known carcinogen. Information on carcinogenicity and toxicity can be found on the MSDS on file in the laboratory. All work with formaldehyde must be done wearing a lab coat, ANSI approved safety glasses, and gloves made of neoprene over rubber, and must be performed under a fume hood. A 40% formaldehyde solution, the concentration of the stock solution, is corrosive and flammable. The 70% ethanol used as a preservative, and the 95% ethanol from which the 70% solution is routinely made, are both flammable

liquids and must be stored in a safety cabinet and used with eye protection.

5.4 Spill procedures: Follow the procedures outlined in the ESC Occupant Emergency Plan (OEP), the Hazardous Material Spills section. For minor spills (which can be handled by the analyst) wear safety glasses, lab coat, and gloves to clean up the material. For major spills, immediately contact the SHEM Manager.

6.0 Equipment and Supplies

6.1 Fume Hood. Fume hood must have running water source (with flexible hose) inside and sufficient air removal to meet OSHA standards for formaldehyde vapors in the air (OSHA Permissible Exposure Limit: 0.75 ppm, Short Term Exposure Limit 2.0 ppm). The hood should also be equipped with a sink or basin.

6.2 Basins and Pans. A large plastic basin (50x42x12cm) is to be used under the hood to capture the initial rinse water when the sample is first removed from the jar. Glass pans (34x22x5cm) are used to sort through the sediment.

6.3 Sieves and funnels. A 500 mesh sieve at least 20 cm in diameter with at least a 6 cm side should be used. Funnels can be of various sizes, but one should have at least a 25 cm diameter opening.

6.4 Illuminated desk magnifying lenses. These light sources are used for picking and should be equipped with a clamp so they can be attached to the bench top. The lens should be about 12 cm in diameter, capable of 1.5 - 3.0x magnification, and surrounded by an approximately 20 cm (8-inch) diameter circular 22W fluorescent bulb.

6.5 Fine Forceps. Fine tipped forceps (comparable to Dumont # 5 forceps) must be used to pick up invertebrates without damaging them.

6.6 Vials and jars. Glass scintillation vials (20ml) with screw caps containing cone-shaped polyethylene liner.

6.7 Label Paper. Waterproof paper, heavy weight such as “Ledger 32" (120 g/m^2) manufactured by “Rite in the Rain”.

6.8 Soft lead pencils.

6.9 Permanent markers.

6.10 Test tube rack.

6.11 Large plastic beakers.

6.12 Disposable plastic pipettes

6.13 Nalgene squirt bottles.

6.14 Petri dishes (various sizes)

7.0 Reagents

7.1 The fixative is 10% (by volume) formaldehyde stained with Rose Bengal (approximately 0.5 g per liter) and buffered with sodium borate added to saturation. Commercially available formaldehyde buffered with sodium phosphate and stabilized with methanol is acceptable (stain can be added as above). Note that the term "formalin" is used in various ways and may not always refer to 10% formaldehyde. The fixative solution should always be prepared in terms of the concentration of formaldehyde, not formalin.

7.2 The preservative is 70% ethanol.

8.0 Sample Collection, Fixation, and Storage

8.1 Estuarine and coastal ocean benthic samples can be collected by a variety of means. The residue of the sample retained on a 0.5 or 1.0 mm mesh sieve is to be fixed with 10% formaldehyde (see 7.1) added to a volume twice that of the material in the jar. Fixation must be allowed to occur for at least 24 hours. Fixed samples can be stored at room temperature.

9.0 Quality Control

9.1 A minimum of 10% of the samples picked by each technician will be re-picked to monitor the effectiveness of the picking process and provide feedback necessary to maintain the minimum acceptable percent effectiveness. Batches of ten samples picked by a technician will be formed at random and one sample randomly selected from each batch and re-picked by another technician. If a batch contains large samples that were picked by two or more technicians (all working on one sample), none of these technicians will participate in the selection or re-picking of the QC sample. The samples comprising each batch, initials of technician(s) who picked the samples, the sample selected to be re-picked, initials of the person(s) who re-picked it, the result of the re-picks, and response actions taken (see below) will be documented in a QC logbook.

9.2 The minimum acceptable degree of picking effectiveness is 90%; however based on experience of other programs using similar methods , picking effectiveness can be expected to be greater than 95%.

9.3 Percent picking effectiveness (PE) will be calculated using the following formula:

$$PE = \frac{\text{\# invertebrates originally picked from sample}}{\text{\# invertebrates originally picked + \# found during re-pick}} \times 100$$

9.4 The results of QC re-picks will determine the action to be taken. If the picking effectiveness is greater than 95%, no action will be required. If it is 90 to 95%, the technician or technicians who picked the QC batch will receive additional instruction on how to improve their effectiveness, giving particular attention to the specific types of animals missed. Laboratory personnel must be particularly sensitive to systematic errors in picking – i.e., the consistent failure to pick specific taxonomic groups--that may suggest the need for additional training. A picking effectiveness below 90% will require re-picking all samples in the batch from which the re-pick sample was selected.

9.5 If re-picking effectiveness is 90% or above, the samples in the QC batch may be disposed in accordance with the benthic waste stream plan. See flow diagram under 17.1.

10.0 Calibration and Standardization

[Not applicable.].

11.0 Procedure

11.1. Removing the fixative from the samples: Turn on hood fan and put on lab coat, eye protection, and rubber gloves. Because samples have been fixed with 10% formaldehyde, care should be taken to avoid breathing vapors or splashing the fixative on face and hands, or in eyes. In most cases, Rose Bengal (a red stain that will stain clothing) has been added to the formaldehyde solution. Select a sample jar and, under the hood with the fan operating, decant the formaldehyde solution through a 0.5 mm mesh sieve resting within the large funnel placed in the top of the formaldehyde holding container. Pour off as much of the formaldehyde solution as possible. Unavoidably some of the sample may be poured off as well, but this should be retained on the sieve sitting in the funnel. The holding container should be kept under the hood at all times. Once the sample is completely sorted, the formaldehyde will be returned to the sample jar to prevent the contents from decomposing.

11.2 Washing sample: Over a large plastic basin under the hood with the fan operating, pour

the remaining contents of the sample jar onto the 0.5 mm mesh sieve. Wash the remainder of the jar contents onto the screen with water. (It may help to rest the sieve on an inverted test tube rack within the basin.) Gently wash the sample contents on the screen with water to remove all formaldehyde from the sample. Wash thoroughly; it is important that all the formaldehyde is removed from the sample at this point, otherwise you may be exposed to vapors while sorting. The wash water should be contained within the basin and poured carefully (using a large funnel) into the 55-gallon drum labeled "Waste formaldehyde" with an estimate of degree of dilution.

11.3 Transferring sample to glass tray. If the sample is small, the entire sample can be washed with tap water from the sieve into a sorting tray. Turn the sieve with the sample upside down over on a tray and, using a minimum of water directed through the screen, wash the sample into the sorting tray. If the sample has a large volume of material, wash it all into a large plastic beaker and transfer small amounts from the beaker to the sorting tray. Once sorted wash the material into a second large plastic beaker which will act as a reservoir of material as tray after tray is sorted. Be sure to label the beakers appropriately, such as "sorted" and "unsorted".

11.4 Picking invertebrates.

11.4.1 Once the sample (or a portion of it) is in a sorting tray, add a small amount of water (1 - 2 cm in depth) and evenly disperse the material to be sorted throughout the tray.

11.4.2 Pick out all large bivalve shells or leaf fragments (if present) and set aside in a petri dish to prevent these objects from possibly blocking the view of some small animals and thereby reducing the efficiency of sorting. (Experience has shown that samples with shells or leaves tend to fail QC more often.) The shells or leaves will be recombined with the sample once it has been sorted. Briefly inspect each shell or leaf fragment to make sure animals are not adhering to it.

11.4.3 Looking through the magnifying lens (equipped with light), scan the contents of the tray systematically from one end to the other, picking out the invertebrates with forceps or a small plastic pipette, and sorting them to major taxonomic categories in vials containing 70% EtOH (stored in flammable liquids closet).

11.5 Sorting and labeling invertebrates.

11.5.1 As the invertebrates are picked from the sample material, sort them into vials filled with 70% ethanol according to the taxonomic categories listed below. Use one vial or jar per category.

Worms - mostly polychaetes, some nemertines (=ribbon worms), and small turbellarians (=flatworm).

Crustaceans - amphipods, isopods, small decapods (=crabs)
Insects - mostly larvae (with practice can be distinguished from crustaceans)
Bivalves - clams, mussels
Gastropods - snails
Misc. - anything that can't be identified as one of the above

Any specimen or fragment of one that cannot be identified to a taxonomic category should be placed in the "Miscellaneous" vial.

11.5.2 As each new taxonomic category is encountered, write an internal label for each vial and place it inside the vial so that it can be read from outside. The internal label should be written clearly in pencil (soft lead) on waterproof paper and should contain the following information:

STATION ID. - Sample #
SIEVE MESH (if more than one used, e.g., 0.5 or 1.0 mm)
DATE (collected)
TAXONOMIC CATEGORY (one of the six listed above)
SORTERS INITIALS

Examples:	C3 - 1	0.5mm	or	F - 3	1.0mm
	1/6 /97			8/15/96	
	Crust.	DR		Worms	DR

The label should not be any longer than the shoulder height of the vial. Really long labels do occasionally wick alcohol out of the vial.

11.5.3 Be sure to fill vial to top with 70% EtOH. If this is not done, one or more specimens may be "stranded" on wall of vial or under cap and consequently dry out, reducing the likelihood they can be identified to species.

11.5.4. If the EtOH in the vial was diluted with water from sorting tray (as often happens when a small pipette is used to pick out animals), decant and replace with new EtOH.

11.5.5 When a sample with a large volume of material is subdivided among two or more sorters, combine vials after the sample is completely picked so that there is only one vial per taxonomic category.

11.5.6 With a permanent marker write on each vial cap the station identification, sample number (example: "C3 - 1"), sieve mesh size, and the first letter of the taxonomic category.

Underline "W" for worms and "M" for Misc. to help distinguish them. This will help speed the process of specimen identification.

11.5.7 Place a rubber band (several if necessary) around all vials from one sample.

11.5.8 Processing and storage of picked sample: Once the sample is completely sorted, return it and the internal label to the original sample jar by first pouring it on to a 0.5 mm sieve (resting on inverted vial rack in large basin) and then washing it into the jar using the wide-mouth funnel. Wash the material into the jar using a minimal amount of tap water. When that is completed, add used formaldehyde from the storage container to the sample jar, a volume about equal to the tap water in the jar, and enough to cover the sediment material.

11.5.9 Once the sample has been returned to the original jar and used formaldehyde added, write on the jar lid: "PICKED BY [your initials]". Record in the sample log that the sample was picked.

11.5.10 Sieve cleaning: Before a sieve is used for another sample, it should be cleaned and back washed thoroughly. The cleaning includes gently scrubbing the upper and lower surfaces of the sieve with a pan brush to remove any organisms stuck on the sieve.

12.0 Data Analysis and Calculations

[Not applicable.]

13.0 Method Performance

[See determination of percent picking effectiveness above.]

14.0 Pollution Prevention

14.1 As described in Section 11.1, the original formaldehyde fixative in a sample jar is saved in a container kept under the hood and reused to preserve the sample residue once it has been picked. The initial washing of the sample, described in Section 11.2, should be done with the minimum amount of water needed to remove the formaldehyde. It should be kept in mind, however, that complete removal of the formaldehyde to reduce technician exposure is extremely important and should take precedence over the need to reduce the volume of waste.

15.0 Waste Management

15.1 Waste: Formaldehyde 10%
Waste Type Code: F003
Amount of Waste/Sample (vol./sample): 5 - 30 L / sample
Treatment: Disposal by contractor. Or, if sample residue in sample jars, return to client.

15.2 All laboratory waste must be handled in accordance with guidelines established in the CHP, the waste and reagent disposal SOP (R3-QA062.0), and the waste stream chart shown in Section 17.1.

16.0 References

16.1 Holland, A.F., A.T. Shaughnessy, L.C. Scott, V.A. Dickens, J.A. Ranasinghe, and J.K. Summers. 1988. Progress report: Long-term benthic monitoring and assessment program for the Maryland portion of Chesapeake Bay (July 1986-October 1987). Prepared for the Maryland DNR PPRP-LTB/EST-88-1.

16.2 Strobel, C.J., D.J. Kemm, L.B. Lobring, J.W. Eichelberger, A. Alford-Stevens, B.B. Potter, R.F. Thomas, J.M. Lazorchak, G.B. Collins, and R.L. Graves. 1995. *Environmental Monitoring and Asssessment Program (EMAP)- Estuaries: Laboratory Methods Manual, Vol.1 - Biological and Physical Analyses.* Office of Research and Development, U.S. Environmental Protection Agency, Narragansett, RI.

17.0 Attachments

17.1 Waste Stream for Benthic Sample Processing